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Construction of a 1-Mb Restriction-Mapped Cosmid Contig Containing the Candidate Region for the Familial Mediterranean Fever Locus (*MEFV*) on Chromosome 16p13.3

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In this paper we describe the assembly and restriction map of a 1.05-Mb cosmid contig spanning the candidate region for familial Mediterranean fever (FMF), a recessively inherited disorder of inflammation localized to 16p13.3. Using a combination of cosmid walking and screening for P1, PAC, BAC, and YAC clones, we have generated a contig of genomic clones spanning ~1050 kb that contains the FMF critical region. The map consists of 179 cosmid, 15 P1, 10 PAC, 3 BAC, and 17 YAC clones, anchored by 27 STS markers. Eight additional STSs have been developed from the ~700 kb immediately centromeric to this genomic region. Five of the 35 STSs are microsatellites that have not been previously reported. *Not*I and *Eco*RI mapping of the overlapping cosmids, hybridization of restriction fragments from cosmids to one another, and STS analyses have been used to validate the assembly of the contig. Our contig totally subsumes the 250-kb interval recently reported, by founder haplotype analysis, to contain the FMF gene. Thus, our high-resolution clone map provides an ideal resource for transcriptional mapping toward the eventual identification of this disease gene. © 1997 Academic Press

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INTRODUCTION

Familial Mediterranean fever (FMF) is a recessively inherited inflammatory disorder occurring primarily in non-Ashkenazi Jews, Armenians, Turks, and Arabs (reviewed in Kastner, 1996). The FMF gene frequency among these populations is very high, with estimates based on family studies as high as 1:10 among some non-Ashkenazi Jewish populations (Daniels *et al.*, 1995) and 1 in 14 among Armenians in Los Angeles (Rogers *et al.*, 1989). The main clinical features include intermittent attacks of fever with abdominal pain, pleurisy, and arthritis of one or more joints; patients are normal between attacks. Patients may also develop systemic amyloidosis, leading to renal failure and death. In the absence of a known biochemical abnormality associated with this disease, our laboratories have established a consortium to identify the gene by positional cloning.

Following a genome-wide linkage analysis in a panel of non-Ashkenazi Jewish families, we localized the gene for FMF (designated *MEFV*) to the short arm of chromosome 16 (Pras *et al.*, 1992). The FMF susceptibility gene maps to the same chromosomal region in Armenians (Shohat *et al.*, 1992), Arabs (Pras *et al.*, 1994), and Turks (Ozen *et al.*, 1996). Subsequent genetic analyses placed *MEFV* between *D16S94* and *D16S80*, a genetic interval of ~9 cM (Aksentijevich *et al.*, 1993a; Fischel-Ghodsian *et al.*, 1993). By the outset of the present physical mapping studies, recombinants

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TABLE 1

STSs from the FMF Candidate Region[a]

Locus	STS name	Location	Primer sequences[b] (5′–3′)	Size (bp)	Ann ten
D16S3365	s310G9_1	c310G9	GTGCTCTCCCAACCTCTCAG AGTGAGCACCTGGGCTTG	121	
D16S3366	s302H7_1	c302H7	CGCAATTAACCCTCACTAAAGG GGGACTCTTCCAGGAGATCC	111	
D16S2844	s54G6_1	c54G6	TCTGGTGGCCCTACGAATTGAG GGGTGGGACTTTAGAATCCGGT	388	
D16S2845	s54G6_2	c54G6	GTCGTTATGCCCGCTGAGTATG CCTAACCTCTCTGGGCCTGTTT	123	
D16S3367	s54G6_3	c54G6	AAGTCCCAAAAGTCACACCG GGAGTTATCAGTGCAGATTACGG	263	
D16S468 (D16S3070) (AC)$_n$	C28(CA)C28(GT)	c363D9 9.8 kb	CCCTCCTTGAATTACTTGAACACGGGA TGGGCTCCCAGGCAGCTCTGAAAGTGGTTT	194–216	
D16S3082 (AC)$_n$	s49B4_1[c]	c49B4 9 kb	TGCGGAAATAACGGTGACACTC TCTCCTCTTATCCTGTACCTCTGCC	118–156	
D16S3368	s334B12_1	c334B12 T7 end	GTGAGCCATCATGCCCAGAGG GTTGCAGTGAGCCAAGACCAC	101	
D16S3369	s406E2_1	c406E2 T3 end	ATTCTGCTCATATGTCACCC GTCGTGGGAAACACGTATAT	118	
D16S3370 (AC)$_n$	s57E1_1	c57E1 T7 end	CTGGCTAGAGAACCACGTAAAC ACTTTGCTAGTGAGGTGATTGG	141–153	
D16S2617 (ATA)$_n$	CHLC.ATA41E08	c414G4 3.5 kb	TGTCCACTAGGTAAAGGCTG TGGGCAACAAGAATGAAATT	93–117	
D16S3371	s414G4_1	c414G4 T7 end	CTAGATAAGGCCACACTGACTGTG GGCCATGAATGAGTATCTTAATAT	210	
D16S3372	s442E2_1	c442E2 T3 end	ATTCAACACCCTTCTTCTGGCCCA GAGCTAGTCAGCTGCATGAGCTGT	181	
D16S3373 (AC)$_n$	s374H9_1	c374H9 4.8 kb	TTTTTAAAAGCAGACTCTGCCC TCTGCATAAATGTTATGCCTGC	183–189	
D16S3275 (AC)$_n$	AFMe034	c360H6 13.3 kb	CAAAGCCCTAAAGTAGCAGT GGGTTTGGAGATTTTTGTAA	198–164	
D16S3374	s273L24_1	PAC 273L24 SP6 end	AACGGAACACAGGAAACACTGG AGAGCAAACCCAATGCGGTG	210	
D16S3375	s231E7_1	iy231E7 Right end	CACCGTGCTTTAACTTGAAT TGAAAAATAGCACTTGAGAATAA	142	
D16S3376 (AC)$_n$	s385D9_1	c385D9 4.1 kb	TTTAATCCTGGGACATTCTTGG ATCAGGAAGGGCTGGGAG	197–227	
D16S3377	s360A1_1	c360A1 T3 end	CTACTGCGCTCATTGCTCAG GTTTGCTGTTCTTGGCCG	153	
D16S3378	s0046C8_1	PAC 0046C8 SP6 end	GACATTTTCAGCGATTGAGAATTA TGTGAGCCACTGCTCCTGGCCATC	156	
D16S3379	s441H9_1	c441H9 T7 end	GAGACAGGGTCTCGCTATGT CTCTTTGTCCACTACTTGCT	221	

within our panel of 65 families defined the candidate interval as tel–*D16S246*–*MEFV*–*D16S2622*, a genetic distance of ~1 cM (Sood *et al.*, 1996). Taking advantage of the founder effect observed in the Moroccan Jewish population (Aksentijevich *et al.*, 1993b), and applying Luria–Delbrück formulas and simulations based on a Poisson branching process, we estimated the gene to be within 0.305 cM of *D16S246* (Levy *et al.*, 1996).

D16S246 lies at the centromeric boundary of the genomic interval from which the polycystic kidney disease gene (*PKD1*) was cloned (Harris *et al.*, 1994), while *D16S2622* is derived from a cosmid (cRT70) that is near the telomeric boundary of the region in which the Rubinstein–Taybi (*RSTS*) gene was found (Petrij *et al.*, 1995). Consequently, the interval between these markers had not been mapped in the course of these two positional cloning projects. Moreover, this region also lacked clonal representation in the integrated physical map of chromosome 16 developed by Doggett *et al.* (1995). By screening a combination of YAC, BAC, PAC, P1, and cosmid libraries, we developed a hi redundant, internally consistent physical map of ~1050-kb region spanning the *MEFV* candidate i val. Microsatellites identified in the process of structing this map have permitted further narro of the interval (J. E. Balow *et al.*, in preparation).

A second FMF consortium comprising several Fr groups recently published data localizing *MEFV* t 250-kb YAC clone 26Fe7 (iy231E7; French FMF sortium, 1996). Our own genetic analysis and the in the present report are in agreement with this ing, but provide a much higher resolution map o region. By placing our own and the French consorti genetic markers on the *Eco*RI restriction map, we created an integrated physical/genetic map of *MEFV* candidate interval.

MATERIALS AND METHODS

YAC libraries and screening. YAC clones were isolated by based screening of several different total human genomic YAC li

TABLE 1—*Continued*

Locus	STS name	Location	Primer sequences[b] (5′–3′)	Size (bp)	Annealing temp (°C)
D16S475 $(AG)_n(AAAG)_n$	s58H4_1[f]	iy58H4 Left end	TGCAGGAATATCGATGGAGTTGG GGAGAGGAAGAGCGATGGGAGTAT	196–232	45
D16S3380	s358B10_1	c358B10 T7 end	GGCACACAGCTTCTCGTGGG GCACTTCGTCCGCCTCTGGGC	91	55
D16S3381	sRT211_1	cRT211	GCAACAGAGCAAGACTCTGC CTGAGGTCACGCAACTAGAGG	129	55
D16S3382 $(AC)_n$	s400C4_1	c400C4	ACTTCTAAGCTCCTGACATGCC ATGATACTTCTGCTGTAAGCGG	202–212	50
D16S3383	s307E6_1	c307E6 T7 end	TGAAAAAGCTGCTCACATC ATTCCTTTTCTGTGCATCC	135	55
D16S2622 $(GATA)_n$	sRT70_1[g]	cRT70	TCACTCTAGCTTGGGTGAAGG CCTCTCCAGAGGACAACTGG	156–168	55
D16S3384	sRT8_1	cRT8	AATGAAGTAAAAAGTGCTTTTGGG CTACAGGGAAGCACTTTGTGG	172	55
D16S3385	sRT4_1	cRT4	ACGGAAGCTATTTGGGGC AAAACCGTAAAACTGAGAGGAGG	169	55
D16S2906	sRT1_1	cRT1	ATTTAACTGATTGGCAGCAGGG GCAACAAACAACACCCTGGAAG	399	55
D16S2907	sRT1_2	cRT1	TGTTTGGAGCCTGTGCTGTTTG CCACTCTGAAAAGAACAGCCGG	103	55
D16S3386	sRT53_1	cRT53	CGCTGGTTCATGGTAACATG AAGTTGAACAGCTGTGTCTAGTGC	222	55
D16S3387	sRT99_1	cRT99	CTCAGCTCAGGATGGTCTCC AGTCCTGTAGGGTAGAGGAGGG	141	55
D16S3388 $(AC)_n$	sN2_6508	cN2	ACAACCCTGCTTACACCCTG GGGAAATTCCATCTCCACAA	143–171	52
D16S3389	sRT163_1	cRT163	CACAGGCACAAGCACCAG AGGCAGAAGGATTACTCGAGG	115	55

[a] Listed in their genomic order, telomeric to centromeric.

[b] In cases where there are multiple primer sets for a given locus, we have listed the primers and conditions used in the present experiments

[c] *D16S468* is the same locus as *D16S3070* (AFMa353yh1). Both map to the same *Eco*RI fragment, and the C28(CA)/C28(GT) forward primer is nt 120–145 of GSDB Accession No. Z53013 (the sequence from which *D16S3070* primers were derived). The reverse primer of C28(CA)/C28(GT) is outside of Z53013.

[d] Primers shown here were developed by screening c49B4 for microsatellites. We subsequently found that s49B4_1 and AFMb070yg5a (*D16S3082*) map to the same *Eco*RI fragment. Comparison of s49B4_1 sequence with that of GSDB Accession No. Z53260 (the sequence from which AFMb070yg5a primers were derived) showed a 96% identity over 272 bp that contained the $(AC)_n$ repeat. Therefore, both sets of primers identify *D16S3082*.

[e] Equivalent to *D16S2618* (CHLC.ATA42A06.P34342). The *D16S2618* forward primer is nt 2–21 of GSDB Accession No. G10253 (the sequence from which *D16S2617* primers were derived). The reverse primer of *D16S2618* is the reverse complement of nt 136–116 of G10253.

[f] Primers shown in this table were derived from end sequence of iy58H4. Later, we found that GSDB sequence L16287 (from which *D16S475* [UT581] primers were derived) is included within this end sequence. The 58H4 forward primer shown here is the reverse complement of nt 382–404 of L16287. The 58H4 reverse primer is outside the limits of L16287. The allele sizes listed here were obtained by digesting the amplification products with *Hin*dII.

[g] sRT70_1 and CHLC.GATA73G05 are both associated with *D16S2622*. The primers shown here were developed by screening cRT70 for microsatellites. On subsequent screening of other new markers reported from the region, we found the CHLC.GATA73G05 primers within the sRT70_1 amplimer sequence.

ies. These included the CEPH megaYAC library (Bellanné-Chantelot *et al.*, 1992; clone names prefixed with *My*), the Washington University YAC A and B libraries (Brownstein *et al.*, 1989; clone names prefixed with *Wy*), and the ICI YAC library (Anand *et al.*, 1990; clone names prefixed with *iy*). Agarose plugs of yeast cells containing total yeast and YAC DNA were prepared (Gemmill *et al.*, 1995) and used to estimate the size of YACs by pulsed-field gel electrophoresis. DNA minipreps of YAC colonies were performed using Puregene kits (Gentra) and were used to confirm STS content mapping and as templates for the generation of inter-*Alu* PCR products (Liu *et al.*, 1993) for hybridization probes.

BAC, PAC, and P1 libraries and screening. BAC clones (Shizuya *et al.*, 1992) were identified by hybridization of cosmid *Eco*RI fragments to high-density gridded human genomic BAC filters purchased from Genome Systems. PAC clones (Ioannou *et al.*, 1994) were identified by PCR-based screening of a pooled human genomic PAC library (Genome Systems) with STSs from the *MEFV* interval. P1 clones (Shepherd *et al.*, 1994) were identified both by PCR-based screening (clones prefixed with *A*) and by hybridization of cosmid restriction fragments to high-density gridded human genomic P1 filters (clones prefixed with *B*).

Cosmid libraries and screening. Cosmid C28, which contains the microsatellite marker C28(CA)/C28(GT) (*D16S468*), was kindly provided by Dr. K. Hayashi (Kyushu University, Tokyo). Cosmids cRT4, cRT8, cRT70, cRT194, cRT197, and cRT211 from the *RSTS* candidate region (Petrij *et al.*, 1995) were kindly provided by Drs. Rachel Giles and Martijn Breuning (Leiden University, The Netherlands), while cosmid cN2 was provided by Dr. Anna Maria Frischauf (Imperial Cancer Research Fund, London). All other cosmids were identified from Los Alamos National Laboratory flow-sorted chromosome-16 cosmid libraries by hybridization to high-density gridded membranes. These included ~4000 fingerprinted cosmids (Stallings *et al.*, 1990, 1992) arrayed on 3 membranes and ~15,000 cosmids (Longmire *et al.*, 1993) arrayed on 10 membranes (library 16-2). Throughout this paper individual cosmid names are prefixed with *c*, and cosmid contigs are prefixed with *C*.

Inter-*Alu* PCR products of YAC, PAC, BAC, and P1 clones, and riboprobes generated from the ends of cosmid clones, were used as hybridization probes after blocking for repetitive sequences using 1000-fold excess of c_0t1 DNA (Life Technologies). Stepwise cosmid walking was performed by generating a T3 or T7 riboprobe from the farthest extending end of the cosmid clone. One microgram of *Hae*III-

digested cosmid DNA was used to generate riboprobe (Maxiscript *in vitro* transcription kit; Ambion) in the presence of 50 μCi of [α-^{32}P]-UTP (specific activity 800 Ci/mmol). DNA from all positive clones was purified from 150-ml cultures using a commercially available kit (Qiagen). One microgram of cosmid DNA was digested with *Eco*RI, separated on 0.8% agarose gels, and blotted onto Gene Screen Plus (Dupont) membranes. The overlap of cosmids (indicated by restriction digest) was verified by hybridization with *Eco*RI fragments of the starting cosmid.

Restriction mapping. Detailed *Eco*RI and *Not*I restriction maps spanning the *MEFV* region were determined by end-labeled restriction mapping of all cosmids with the oligo mapping method described in Evans *et al.* (1989). Briefly, 4 μg of cosmid DNA was digested with *Not*I, and an aliquot (1 μg) was saved to detect internal *Not*I sites. To the remainder of *Not*I-digested DNA, 5 μl of a 1/100 dilution of *Eco*RI (10 U/μl) in 1× buffer was added and incubated at 37°C. Aliquots of 1 μg each were removed after 1 and 5 min of incubation, and digestion of the remaining DNA was carried to completion by adding 1 μl of *Eco*RI. In addition, 1 μg of cosmid DNA was digested with *Eco*RI alone to localize the *Not*I sites within *Eco*RI fragments. All samples were separated on 0.8% agarose gels, blotted onto Gene Screen Plus membranes, and hybridized with ^{32}P-labeled T3 and T7 oligonucleotides sequentially. The DNA Analysis Marker System (Life Technologies) was used to estimate the size of the partial fragments hybridizing to the T3 and T7 oligonucleotides.

Probe labeling and hybridizations. Oligonucleotide probes were end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP and were passed through G25-Sephadex spin columns (5 Prime–3 Prime) to remove the unincorporated nucleotides. Inter-*Alu* PCR probes and *Eco*RI fragments from cosmids were labeled using a random-priming kit (Stratagene) and [α-^{32}P]dCTP. All probes except oligonucleotides were blocked with an excess of c_0t1 DNA prior to hybridizations. All Southern blot hybridizations were performed in Rapid-Hyb buffer (Amersham Life Science). The blots were prehybridized for 15–30 min, hybridized for 1–2 h, and washed twice at room temperature in 2× SSC, 0.1% SDS followed by washing at 65°C in 0.1× SSC, 0.1% SDS. For gridded cosmid libraries, hybridizations were performed overnight in 0.5 *M* sodium phosphate (pH 7.2), 7% SDS, 1 m*M* EDTA, followed by washes as described above.

Generation of STSs. (i) End-clone STSs: T3 or T7 ends of cosmids were sequenced to generate a number of STSs for screening of large-insert clone libraries. End fragments of YAC clones iy58H4 (7Dh4) and iy231E7 (26Fe7) and of PAC 273L24 were obtained by bubble PCR using linkers complementary to *Hae*III and *Rsa*I sites (Riley *et al.*, 1990). Oligonucleotide primers were selected with the aid of the computer program PRIMER (Whitehead Institute) or ASTS (Rappaport *et al.*, 1994). PCR conditions for all STSs are described in Table 1. Chromosome-16-specific breakpoint hybrids CY18, CY14, CY186, 23HA, and CY190 (Callen *et al.*, 1992) were screened to confirm the mapping of the STSs to the *MEFV* interval within 16p13.3.

(ii) Microsatellite-associated STSs: Cosmids in the minimal tiling path were screened for dinucleotide repeats to develop new genetic markers from the region. In addition, *D16S468* (Iizuka *et al.*, 1993) and *D16S2617* were found to map to the *MEFV* region by recombinant analysis in FMF families (J. E. Balow *et al.*, in preparation). *D16S3275* had previously been isolated from iy231E7 (26Fe7; French FMF Consortium, 1996) and was localized within cosmid 360H6 by oligonucleotide hybridization.

Fluorescence in situ hybridization (FISH). Cosmids c383C12 and cRT70 were labeled with biotin-14-dATP (BioNick kit; Life Technologies) and digoxigenin-11-dUTP (Boehringer Manheim), respectively, using a nick-translation kit (Life Technologies). Two separate labeling reactions using both of the above-mentioned systems were performed on c57E1, and the reactions were mixed prior to hybridization. EBV-transformed lymphoblastoid cell cultures from a normal male were treated with ethidium bromide followed by colcemid and KCl treatment for metaphase preparation. Dual-color FISH conditions were as described in Trask *et al.* (1991) with minor modifications; signal detection and amplification were done using the Signal Amplification Reagent Set (Oncor). Slides were visualized a Zeiss axiophot microscope with a dual-pass filter.

RESULTS

Attempts to Construct a YAC Contig of the FMF Candidate Region

A number of CEPH megaYAC clones were previo mapped to 16p13.3 near the vicinity of *MEFV* (Chu kov *et al.*, 1995; Doggett *et al.*, 1995). These were te for *D16S2844*, *D16S2845*, and *D16S3367* from c5 (which contains the RFLP *D16S246*) and the cR tetranucleotide repeat *D16S2622*. In addition, S were developed from cosmids that overlapped cR on the centromeric side of *MEFV* (Table 1). Most o YACs that mapped to 16p13.3 were excluded from region because they were negative for these STSs. F YACs, My655E10, My716D1, My641C2, and My64 were positive for *D16S2622* and centromeric STSs negative for the c54G6 STSs. Only one YAC cl My806D3, was positive for markers on both ends o interval. However, this YAC was found to be unst since (i) 20 separate cultures each yielded differ sized YACs, ranging from 1 to 1.7 Mb and (ii) inter-PCR products of this YAC hybridized to far fewer t the expected number of chromosome-16 cosmids f YAC of expected size 1 to 1.7 Mb. The CEPH mega library was also screened commercially with *D16S3* and *D16S3385* primers, which were derived f c54G6 and cRT4, respectively. No clones were ide fied with *D16S3367* primers, while My641C2 My716D1 were selected by *D16S3385* primers. Bo these YACs were later found to extend in the cen meric direction away from the *MEFV* locus.

Due to the problems encountered with CEPH m YACs from the region, we also screened smaller in YAC libraries. The overall strategy we employed physical mapping is shown in Fig. 1. Briefly, cos walking led to the development of new STSs, w were used to screen the ICI and Washington Univer YAC libraries. A total of 17 YAC clones were identi from these libraries (Fig. 2), with insert sizes ran from 150 to 440 kb. Even though we did not comp a YAC contig of the region, these smaller YACs w very useful as probes for the cosmid library screen and in this manner contributed to the assembly of cosmid contig.

Identification of BACs, PACs, and P1 Clones

A gridded BAC library became available only tow the end of the physical mapping project. BAC fil were screened with a 1.9-kb *Eco*RI fragment of c44 and an SP6 (centromeric) end-clone probe from F 273L24 to facilitate closure of the final gap in the mid contig (see below). Five positive clones were ide fied with the c441H9 fragment, one of which, F 36P15, extended in the telomeric direction and cluded a region in which cosmids were found to cont

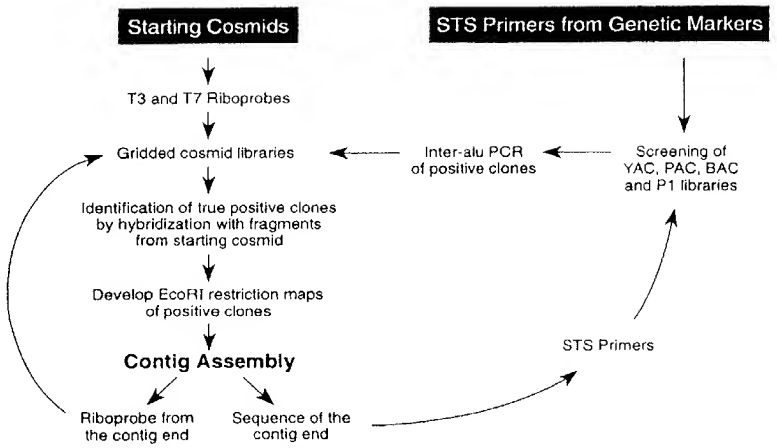


FIG. 1. Strategy used in cloning and physical mapping of the FMF candidate region. The starting cosmids were c54G6 (containing the marker *D16S246*) and cRT70 (containing the marker *D16S2622*).

internal deletions. Using DNA from this BAC, we were able to estimate the size of this deleted region and to deduce its *Eco*RI restriction pattern. BAC 153H7 was positive for the SP6 end of PAC 273L24 and extended just up to the deletion area (Fig. 2).

Ten PAC clones were identified by screening with eight sets of primer pairs (Fig. 2). PCR- and hybridization-based P1 library screening identified 15 clones (Fig. 2). Of these, P1 B53E2 linked c54G6 to cosmids from contig 387 (which was known to contain the distal marker *D16S94*) and thereby helped orient the cosmid walk from this end.

Construction of the Cosmid Contig

A cosmid contig for the FMF region was generated by stepwise cosmid walking using riboprobes from the ends of cosmids and inter-*Alu* PCR probes from P1, PAC, BAC, and YAC clones, against both the fingerprinted (1.5×) and the 16-2 (5.5×) chromosome-16 cosmid libraries. The initial attempts at cosmid walking were made in the fingerprinted library. This library provided 58% coverage of chromosome 16 in cosmid contigs and an additional 28% in fingerprinted singleton clones (Stallings *et al.*, 1992). The starting points for walking in this library were the cosmids containing the flanking genetic markers, i.e., c54G6 (*D16S246*) and cRT70 (*D16S2622*). At every step, overlaps were detected by hybridization of fragments from the starting cosmid and confirmed by detailed *Eco*RI and *Not*I restriction maps of each cosmid (Fig. 3).

The first round of screening with cosmids c54G6 and cRT70 from each end of the interval was performed bidirectionally, using both T3 and T7 riboprobes, since the orientation of these clones was not known. We were able to orient the walk at cRT70 by finding overlap of T7–cRT70-positive clones with cRT8, which was known to extend in the centromeric direction (Petrij *et al.*, 1995), and therefore walking was continued only from the T3 end of cRT70 toward the telomere. We were unable to identify any cosmids overlapping with c54G6 from the 1.5× fingerprinted cosmid library. In subsequent walking experiments, gridded membranes of the 5.5× library were also used. We were able to identify several overlapping cosmids with c54G6 from this library. The orientation of the walk from this end was established by showing that T3–c54G6-positive clones overlapped with P1 B53E2, which had been determined to extend distally from c54G6. Thus, cosmids identified from the T7 end of c54G6 extended centromeric across the FMF interval.

As cosmid contig assembly was progressing from both ends of the candidate interval, *D16S468* was localized to the region by analysis of DNA from a somatic cell hybrid breakpoint mapping panel (Callen *et al.*, 1992, 1995). Analysis of recombinants placed this marker between *D16S246* and *D16S2622*, telomeric to *MEFV* (J. E. Balow *et al.*, in preparation). Two cosmids, c363D9 and c420A3, were identified by screening cosmid libraries with oligonucleotides for *D16S468*, and cosmid C28 (from which the *D16S468* sequence was originally derived) was obtained from Dr. K. Hayashi. These three overlapping cosmids formed a small contig between the flanking markers for *MEFV* and were used as another nucleation point for cosmid walking. After extending this contig to c383C12 in one direction, and to c57E1 in the other direction, dual-color FISH analysis (Fig. 4) was performed to determine the relative order of these cosmids. Additional walks were initiated at cRT194 and cRT211 (cosmids provided by Dr. Breuning that were mapped by FISH telomeric of cRT70).

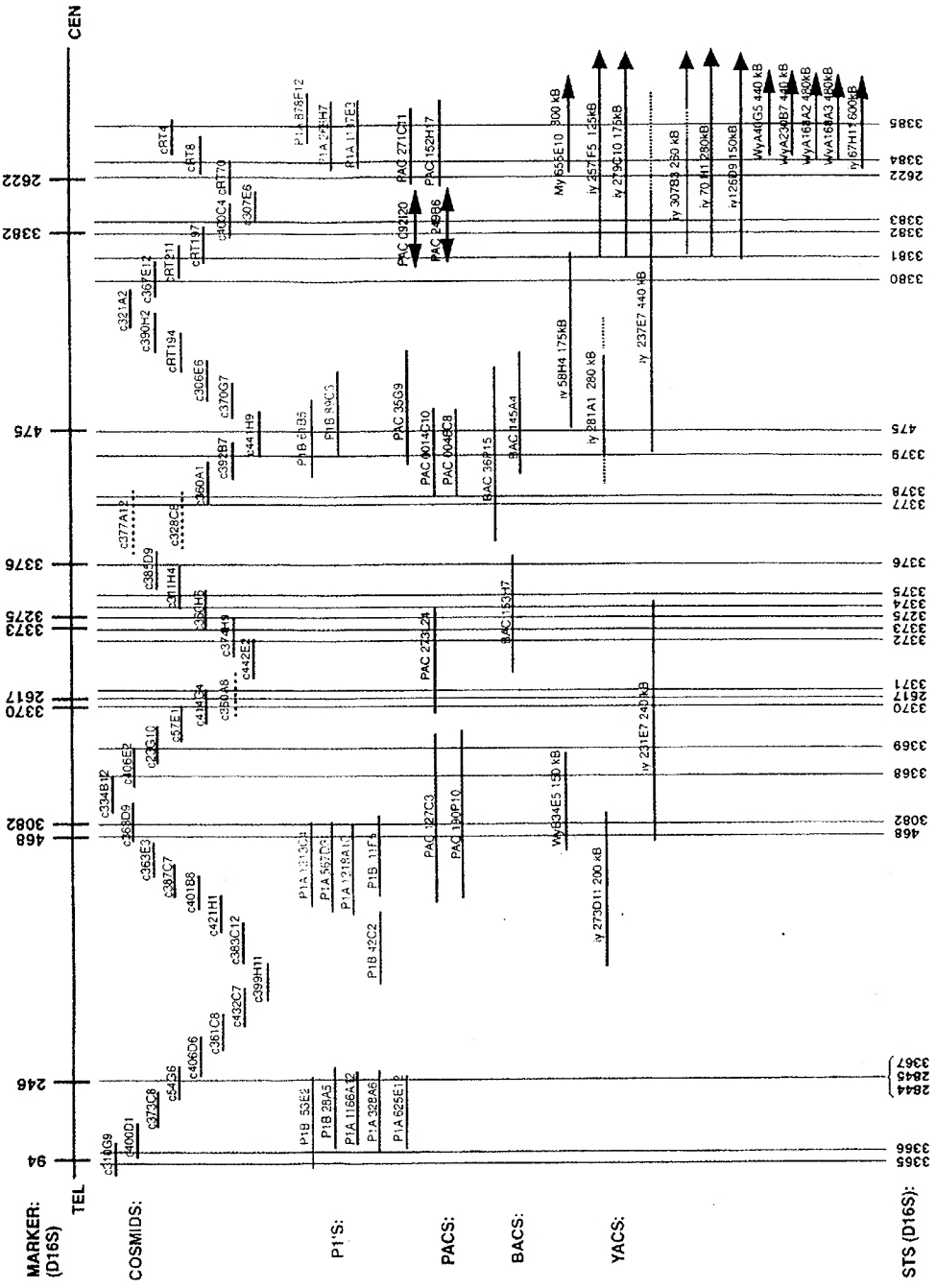


FIG. 2. A contig of overlapping cosmid, P1, PAC, BAC, and YAC clones from the FMF region showing the locations of 12 genetic (short vertical lines) and 29 STS (long vertical lines) markers. The map is drawn with a minimal set of cosmid clones. Horizontal lines represent the extent of each clone. Deletions observed in various clones are shown by dashed lines. Alternate designations for ICI YACs are as follows: iy58H4, 7Dh4; iy67H11, 8Dh11; iy70H1, 8Gh1; iy126D9, 14H9; iy231E7, 26Fe7; iy237E7, 27Ca7; iy257F5, 29E5; iy273D11, 31Cd11; iy279C10, 31Ic10, iy281A1.

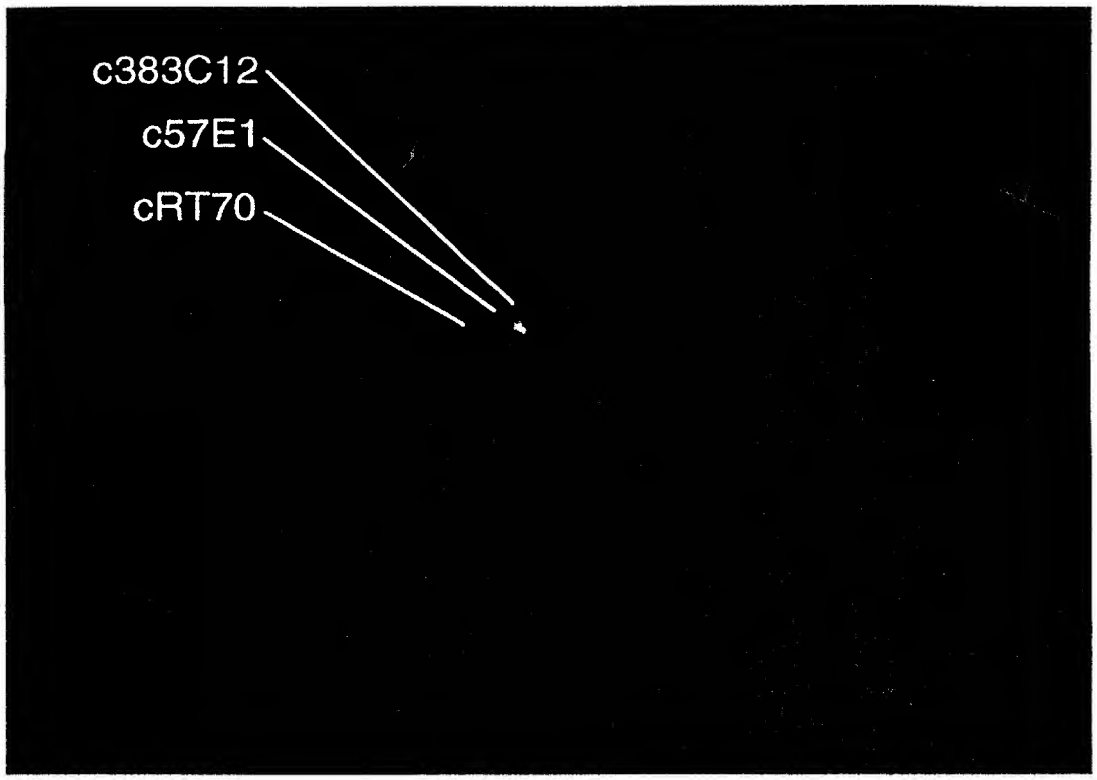


FIG. 4. Determination of the orientation of the contig generated at c363D9 by dual-color FISH analysis. c383C12 (red) is telomeric to both c57E1 (yellow) and cRT70 (green). This order was detected in 37/52 (71%) of chromosomes analyzed.

In all, both chromosome-16 cosmid libraries were screened with 26 riboprobes; 14 inter-*Alu* PCR products of P1, PAC, BAC, and YAC clones; one pair of oligonucleotides for the marker *D16S468;* and DNA probes derived from the SP6 end of PAC 273L24 and the right (centromeric) end of iy231E7 (26Fe7). These hybridizations identified 474 cosmid clones, of which 179 mapped to the FMF candidate region, providing a continuous coverage of approximately 1050 kb. Only 42 clones were analyzed from the fingerprinted library, of which 23 mapped in the region. Twelve clones belonged to four previously characterized contigs, C211, C330, C664, and C387 (Doggett *et al.,* 1995). The remaining 11 cosmids identified in this library were singleton clones, nonoverlapping with cosmids in the previously available chromosome-16 physical map.

Based on the nature of the libraries used, we expected to achieve six- to sevenfold coverage of the region by cosmids. Although that turned out to be the case for most of the contig, areas of both underrepresentation and overrepresentation were detected throughout the contig. It was particularly important to confirm overlaps in the underrepresented regions where small stretches of DNA at the ends of cosmids were minimally overlapping between only two cosmid clones (Fig. 3). These overlaps were confirmed by hybridization of end fragments to each other and to common fragments of overlapping P1, PAC, and YAC clones. To estimate the size of the corresponding *Eco*RI fragment in genomic DNA, the degree of overlap between the end fragments was used for the overlap of the T3 end of c400D1 with the T3 end of c373C8. Restriction digests from YAC WyB34E5 were sized to determine the *Eco*RI fragment at the overlap of the T3 end of c363E3 with the T3 end of c363D9. Restriction digests from PAC clone 273L24 were used to determine the *Eco*RI fragments at the overlap of the T7 end of c360A8 with the T7 end of c442E2/c442F3 and of the T7 end of c374H9 with the T3 end of c360H6.

Deletions and Instability in Cosmids

Although cosmids are relatively stable cloning vehicles, by detailed analysis of 179 clones encompassing this 1.05-Mb interval we found five cosmids from three separate regions with deletions. In one of these regions, two overlapping cosmids, c328C8 and c377A12, were unstable in culture. The deleted area in these cosmids is shown by a dashed line in Fig. 3. We were unable to identify any stable cosmid that spanned this region. By analyzing BAC 36P15 DNA, we estimated the extent of the deletion to be ~30 kb, with *Eco*RI fragments of ~16, 7.5, 3.6, and 2.8 kb. Moreover, as is predicted from the location of *Not*I sites in cosmids that flank the deleted region, pulsed-field genomic Southern blots demonstrated an ~170-kb *Not*I fragment when hybridized to a probe derived from c441H9 (data not shown), providing further evidence that BAC 36P15 contained an intact set of *Eco*RI fragments from this region.

In a second region about 125 kb telomeric to this first unstable area, we found two overlapping cosmids (c360A8 and c373H9) that showed two different deletions relative to other cosmids in the region. In contrast to the c377A12/c328C8 segment, there are several stable cosmids spanning this second region. The extent of the deletions in c360A8 and c373H9 was determined by hybridization of individual *Eco*RI fragments from c347C2 and c414G4. We estimated that ~24 kb of genomic DNA had been deleted from c373H9 and 6.3 kb from c360A8. Multiple cultures of c360A8 and c373H9 yielded reproducible restriction maps, indicating that the deletions observed in these cosmids occurred early in the library construction.

Finally, in a region about 150 kb further telomeric, we found that cosmid C28 bears a deletion relative to other cosmids in the region. However, the significance of this observation is unclear, given the fact that this cosmid is derived from a different library, with a different donor of genomic DNA.

Integration of Physical and Genetic Maps

In the course of constructing this map, we attempted to determine the physical locations of existing genetic landmarks and to identify new polymorphic markers from the region. At the telomeric end of the interval, the $(AC)_n$ repeat *D16S94* (VK5) has been mapped to the 12-kb *Eco*RI fragment of c310G9, while the Southern blot marker *D16S246* (p218EP6) has been localized within c54G6. As is noted above, *D16S468* maps to c363D9, and this information was used to establish a nucleation point for cosmid walking. We subsequer found that *D16S3070* maps to the same *Eco*RI fr ment as does *D16S468*, and sequence comparisons tween *D16S468* primers and the *D16S3070* amplii confirm that these are the same locus. Approxima 150 kb centromeric to *D16S468*, CHLC microsatel *D16S2617* was mapped to the 3.5-kb *Eco*RI fragm of c414G4. *D16S3275*, which was recently reporte be the centromeric end of the candidate interval defi by founder haplotypes (French FMF Consorti 1996), is approximately 75 kb further centrome within the 13.3-kb *Eco*RI fragment of c360H6.

In the course of screening our clones for microsa lites, we identified three polymorphisms that were dependently identified by others. At the left (telomc end of iy58H4 (7Dh4), we identified a complex rep that, by sequence and map location, is the same *D16S475*. In c49B4, about 20 kb centromeric *D16S468/D16S3070*, we identified an $(AC)_n$ repeat t is the same as Généthon marker *D16S3082*. Finall tetranucleotide repeat polymorphism that we had veloped from cRT70 is the same as the CHLC mar *D16S2622*.

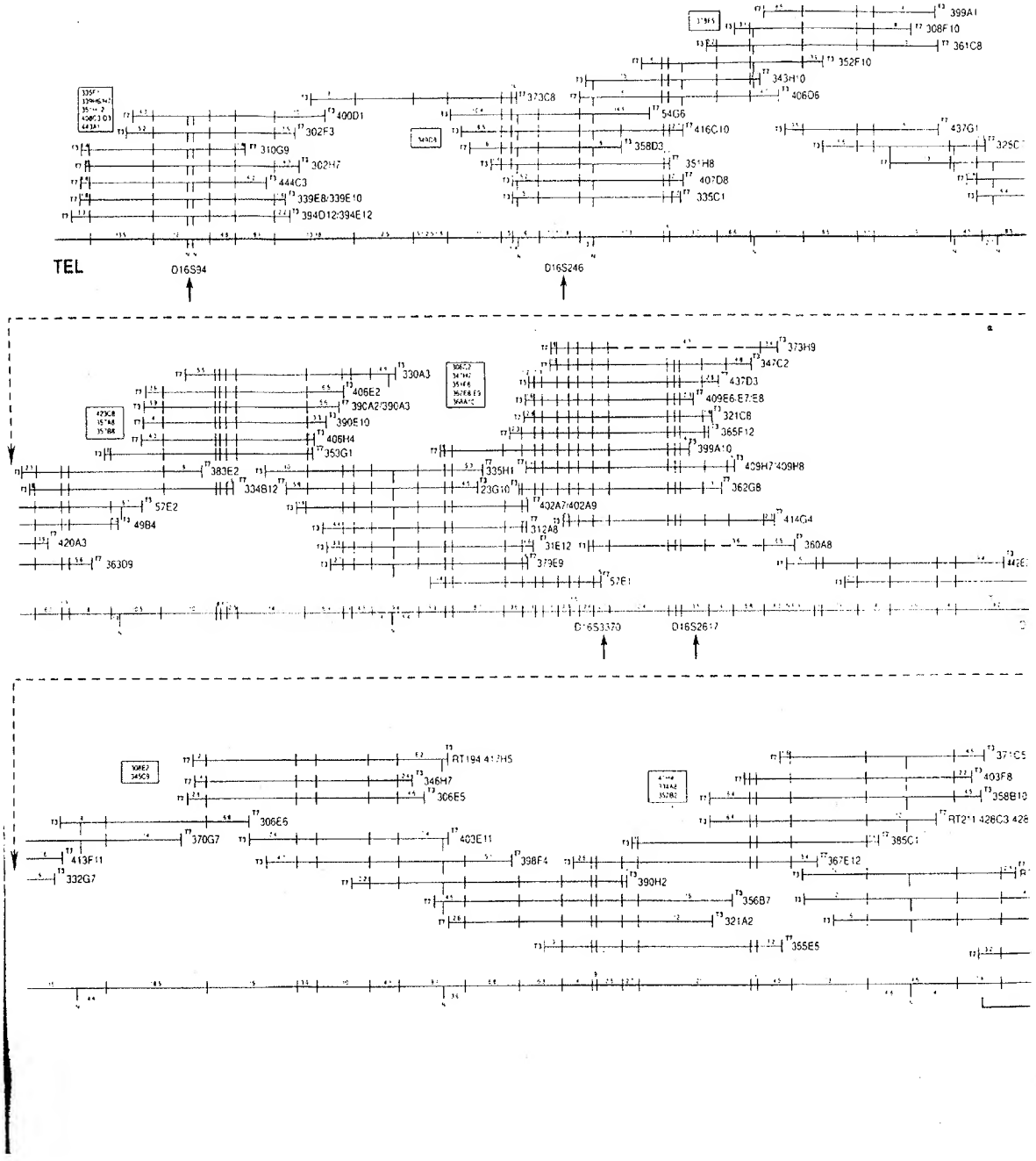
In addition, we identified five microsatellites t have not been previously reported. These *D16S3370*, *D16S3373*, *D16S3376*, *D16S3382*, *D16S3388*. The first four have all been mapped wit our cosmid contig, while *D16S3388* is derived from c approximately 700 kb centromeric to cRT70 (Petri *al.*, 1995).

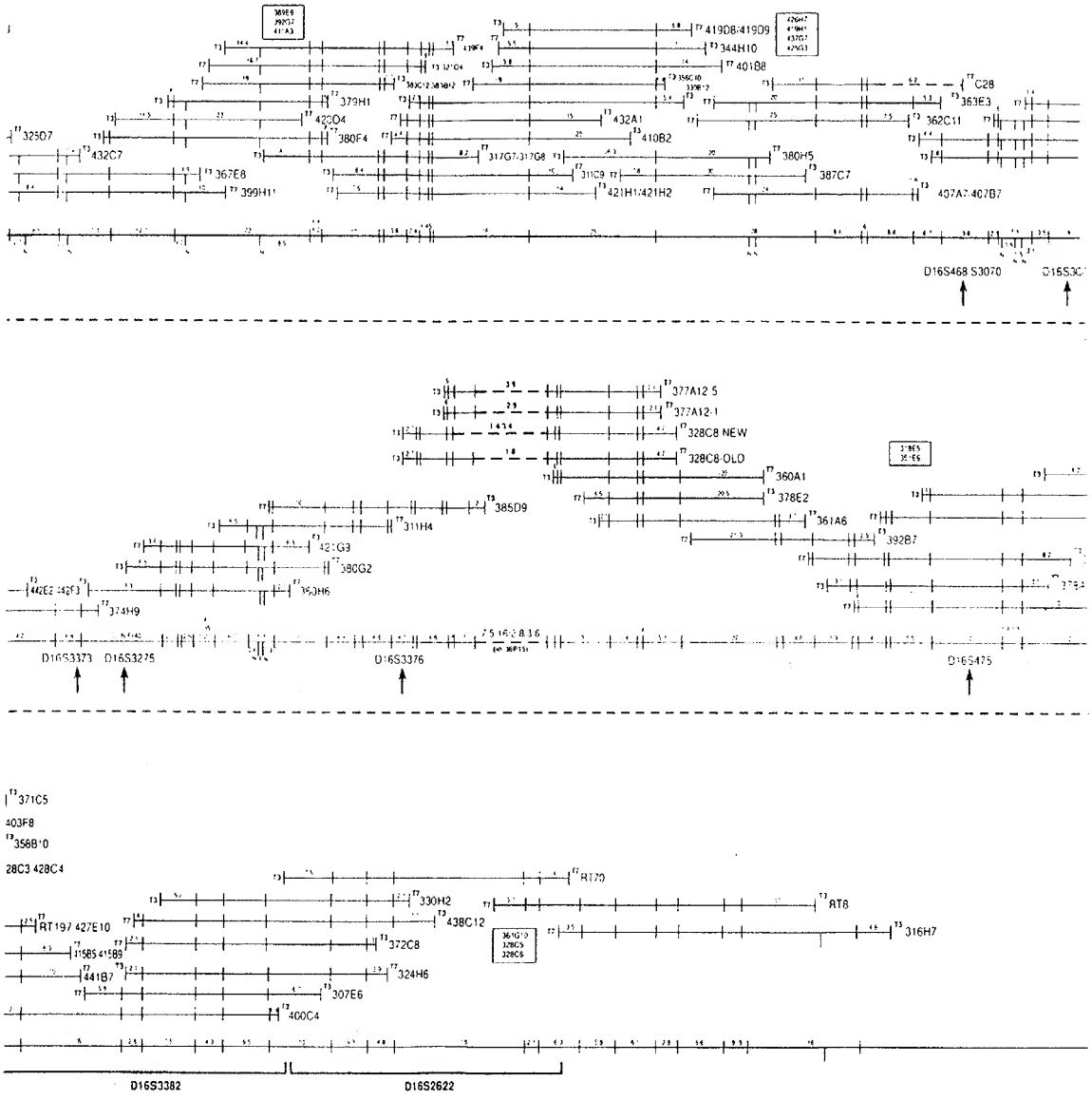
The boundaries of the FMF candidate interval fined by non-Ashkenazi Jewish founder haplotypes *D16S468/D16S3070* and *D16S3275* (French FMF C sortium, 1996). Historical recombinants in our pa of families slightly narrow the candidate interval to region between *D16S3082* and *D16S3373* (J. E. Ba *et al.*, in preparation). Depending on their exact p tions within their respective *Eco*RI fragments, th markers are 190–205 kb apart.

DISCUSSION

In this article we present a clone-based, high-res tion physical map of approximately 1 Mb of distal ch mosome 16p. The cloned interval includes the car date region for *MEFV*, the familial Mediterranean ver locus, and physically links extensive maps t have already been constructed distally in the polycy kidney disease (*PKD1*)/tuberous sclerosis (TSC2) gion (Dackowski *et al.*, 1996) and proximally in

FIG. 3. Detailed *Eco*RI and *Not*I restriction map of the ~1050-kb cosmid contig spanning the interval between *D16S94* and *D16S2* Cosmids are represented by horizontal lines with T3 and T7 ends marked on either side. In areas of overrepresentation, not all cosr were restriction-mapped, and such cosmids are listed in boxes at the appropriate position in the contig. Genomic DNA is represented continuous horizontal line with its orientation relative to the chromosome indicated at the two ends. *Eco*RI sites are indicated by ver lines going through the horizontal lines, whereas *Not*I sites are shown as lower vertical lines and an N. Numbers above the DNA line *Eco*RI fragment sizes; numbers below are *Not*I–*Eco*RI or *Not*I–*Not*I fragment sizes. A shill (/) is used to list multiple *Eco*RI sites if t relative order could not be determined. Deletions in cosmids are shown by dashed lines. Fragment sizes estimated from PAC273L24 denoted as "6140".


399A1
308F10
361C8
352F10
343H10
406D6
373C8
54G6
416C10
437G1
400D1
302F3
310G9
302H7
444C3
339E8/339E10
394D12/394E12
358D3
351H8
407D8
335C1
TEL
D16S94
D16S246
373H9
347C2
437D3
409E6-E7/E8
321C8
365F12
399A10
409H7/409H8
362G8
414G4
360A8
330A3
406E2
390A2/390A3
390E10
406H4
353G1
383E2
334B12
335H1
23G10
402A7/402A9
312A8
31E12
379E9
52E1
52E2
49B4
420A3
36309
D16S3370
D16S2617
RT194 413H5
346H7
306E5
306E6
370G7
403E11
413F11
332G7
398F4
390H2
356B7
321A2
355E5
371C5
403F8
358B10
RT211 428C3 428
385C1
367E12


419D8/419D9
344H10
401B8
C28
363E3
362C11
379H1
420D4
432A1
325D7
380F4
410B2
432C7
380H5
367E8
387C7
399H11
D16S468
S3070
377A12.5
377A12.1
328C8 NEW
328C8 OLD
360A1
378E2
385D9
311H4
361A6
392B7
421G3
380G2
374H9
D16S3373
D16S3275
D16S3376
D16S475

371C5
403F8
356B10
28C3 428C4
RT70
330H2
RT8
RT197 427E10
438C12
316H7
372C8
324H6
441B7
307E6
400C4
D16S3382
D16S2622

Rubinstein–Taybi (*RSTS*)/CREB-binding protein (*CBP*) region (Giles *et al.*, 1996). *In toto*, this completes approximately 4 Mb of continuous sequence-ready maps of band 16p13.3 (Doggett *et al.*, 1996). In the context of the chromosome-16 mouse/human somatic cell hybrid breakpoint map (Callen *et al.*, 1992, 1995), our 1.05-Mb contig completely spans the CY190–CY186/23HA interval, with *D16S94* (VK5) distal to the CY190–CY186 breakpoint and *D16S3381* and *D16S3383* proximal to 23HA (data not shown). Presumably, normal sequences from both breakpoints are present in our contig.

Within the 1.05-Mb cloned interval we have mapped 27 STSs, including 18 markers not previously reported. This gives an average marker density of approximately 1 STS every 40 kb, substantially better than the genome-wide goal of 1 STS/100 kb. Moreover, within the region between *D16S468/D16S3070* and *D16S475*, the density is 1 STS every 23 kb. We have also developed an additional 8 STSs from the ~700-kb interval centromeric to cRT70 (*D16S2622*).

Of the 27 STSs localized to the FMF region cosmid contig, 10 are polymorphic microsatellites, and 4 (*D16S3370, D16S3373, D16S3376,* and *D16S3382*) have not been described prior to the present report. Two of these latter microsatellites fall within the 250-kb FMF critical region recently defined on the basis of founder haplotypes (French FMF Consortium, 1996). We have also identified a fifth microsatellite (*D16S3388*) from the more centromeric cosmid cN2.

In addition, we found a second microsatellite from cN2 that, based on the sequence of the respective amplimers, is the same locus as the Généthon marker *D16S3072* (AFMb015wa9). Thus, our directed search for microsatellites in this region identified a total of four polymorphisms (*D16S3082, D16S475, D16S2622,* and *D16S3072*) that had also been found through genome-wide microsatellite mapping projects. Moreover, based on map location and primer sequence, we found that *D16S3070* (AFMa353yh1) is the same as *D16S468* (C28). Map location was important in raising our index of suspicion in this latter case, since amplimer sequence is not available in the databases for *D16S468*. Our experience suggests that ascertainment of di-, tri-, and tetranucleotide polymorphisms from this region may be relatively complete and emphasizes the utility of a detailed physical map in evaluating the uniqueness of newly identified markers.

Based on CEPH family genotyping, the sex-averaged genetic distance between *D16S246* and *D16S468/S3070* is ~1 cM, while the genetic distance between the latter locus and *D16S475* is ~2 cM (French FMF Consortium, 1996). On our map, the physical distance between *D16S246* and *D16S475* is approximately 635 kb, indicating an approximate physical/genetic distance ratio of 200 kb/cM. The whole chromosome average for chromosome 16 is 95 Mb/152 cM or 600 kb/cM (Kozman *et al.*, 1995). It therefore appears that the FMF region is a "hot spot" of recombination relative to the rest of chromosome 16 and markedly so relative to the genome-wide average of 1000 kb/cM. Since the physical distance from *D16S246* to *D16S468/S3070* is roughly half the physical distance from *D16S468/S3070* to *D16S475*, the physical/genetic distance ratio is relatively uniform over the interval, to the present level of resolution.

In addition to being relatively recombinogenic in man, this 1-Mb region is relatively unstable as large-insert YACs. Similarly, Doggett *et al.* (1995) have reported an apparent lack of megaYAC clones in the distal 2.85 Mb of 16p13.3 (telomeric to *MEFV*), while instability of YACs in proximal 16p13.3 (centromeric to *MEFV*) led Petrij *et al.* (1995) to use cosmids in their hunt for the Rubinstein–Taybi gene. Taken together, these data imply that the distal ~4 Mb of 16p is relatively unstable/underrepresented in megaYACs. We were, however, able to identify stable smaller-insert YACs from the ICI library in both the proximal and the distal regions of our contig, while the central region of the contig, between iy231E7 (26Fe7) and iy237E7 (27Ce7), was not represented. It is intriguing to note that we also observed instability and relative underrepresentation of cosmids in this same central region. Thus, there may be sequences in this particular region that impair stable propagation in both yeast and bacterial systems. The failure to find any stable megaYACs spanning the interval may be due to the fact that any such clone would necessarily contain these sequences. Based on data from the *PKD1* region, one can speculate that instability may be due to the presence of repeats and duplications and/or a high GC content. It is unlikely that the instability in YACs is directly related to the high recombination frequency observed across this interval, since another hot spot at 16p12 is well represented in megaYACs (Callen *et al.*, 1995).

The utility of BAC clones in spanning this unstable region suggests that low copy number may be important in propagating these sequences in bacterial systems. The close agreement of physical distance estimates based on BAC clones and pulsed-field mapping of genomic DNA argues against any major additional gaps in our map. In addition, this experience once again underscores the importance of employing several complementary cloning systems in constructing an accurate map of this magnitude.

Of course, the *raison d'être* for this map is the identification, by positional cloning, of the gene causing FMF. We are currently employing exon-trapping, direct cDNA selection, and single-pass sequencing to develop a detailed transcriptional map of the region. Given that distal 16p is relatively gene dense, it is likely that the resources created here will be of use in other cloning projects, as well.

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Note added in proof. Upon analysis of the sequence generated from c399A10, we found an *Eco*RI fragment of 3.1 kb that is not shown in Fig. 3. This fragment lies between the 3.6- and the 3-kb fragments about 15 kb telomeric to the marker *D16S3370*. The fragment was not detected by original analysis due to the presence of two other fragments of the same size in this region.

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